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AWARD NUMBER DAMD17-97-1-7288

TITLE: Dual-Specificity Anit-HER-2/neu Antisense DNA Agents for Breast Cancer Therapy

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REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 98 - 30 Jun 99)	
4. TITLE AND SUBTITLE Dual-Specificity Anit-HER-2/neu Antisense DNA Agents for Breast Cancer Therapy			5. FUNDING NUMBERS DAMD17-97-1-7288	
6. AUTHOR(S) Stanley Stein, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Medicine and Dentistry of New Jersey Piscataway, New Jersey 08854			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The goal of the first phase of this project, which constitutes the major technological challenge to be overcome, is to design a peptidic compound capable of binding to HER-2/neu mRNA in the presence of a large excess of unrelated RNA. To accomplish this goal, we have devised a deconvolution strategy based on synthesis of arrays of defined cationic peptides on SPOTS membranes, followed by screening with radiolabeled HER-2/neu RNA in the presence of a large excess of tRNA. This process appears to be working satisfactorily after 3 cycles of deconvolution. This deconvolution process should be completed in the fall. Methodology for the next phase of the project, namely conjugation of a cationic peptide to an oligonucleotide and RNase H cleave assay with this conjugate has also been developed in anticipation of the final deconvoluted peptide.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 20	
HER-2/neu, peptide deconvolution, combinatorial library, RNase H			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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INTRODUCTION

This research program concerns the development of a therapeutic agent that can specifically inhibit proliferation of HER-2/neu overexpressing breast cancer cells. The purpose of the experimentation is to use a combinatorial chemistry approach to select a peptidic compound that can selectively recognize and strongly bind to a unique 3-dimensional folded site on HER-2/neu mRNA. This peptidic compound will then be used to target an appended antisense DNA to HER-2/neu mRNA, thereby inhibiting production of the gene product, which is presumed to be responsible for cellular proliferation. The subject matter of this report is designing and using a novel method for selection of a specific peptide for selective and strong HER-2/neu mRNA binding.

BODY

Tasks 1 - 4:

The research program in the grant application listed 4 Tasks for the first 18 months. Together, these Tasks involved designing and applying chemical procedures that will result in a unique compound. This unique compound will have the property of being able to bind to a folded region of HER-2/neu mRNA, both specifically and with high affinity. Accomplishing this feat is a considerable challenge for 2 reasons. First, HER-2/neu mRNA will comprise but a minute portion of the total RNA (i.e. other mRNAs, ribosomal RNA, tRNA, etc.). Second, RNA in general can interact with other chemicals due to its having a negatively charged backbone for ionic interactions, as well as aromatic bases that can participate in both hydrogen bonds and hydrophobic interactions. However, the therapeutic compound to be developed does not have to depend only on its ability to bind HER-2/neu mRNA, because it will be linked to an antisense DNA. Accordingly, the therapeutic activity will be specific for HER-2/neu mRNA because both the peptidic compound and the antisense DNA, which will be linked together, must both simultaneously recognize and bind to HER-2/neu mRNA, as opposed to any other RNA in the cell. Furthermore, our selection process focuses first on the peptidic portion of the anti-HER-2/neu mRNA compound, which therefore does not have to have extraordinary specificity for the target HER-2/neu mRNA. Extraordinary specificity will be gained later when the selected peptidic compound is linked to a selected antisense DNA.

Synthesis and selection of the peptidic compound:

As reported last year, we have been able to prepare highly pure radiolabeled HER-2/neu mRNA for use in screening. We also reported on the development of a process for synthesizing PEG-peptide conjugates. After careful consideration, we decided to modify our approach for selecting the desired HER-2/neu mRNA-binding peptidic compound. The original

procedure was to extract a specific PEG-peptide from the synthetic combinatorial library of PEG-peptides using HER-2/neu mRNA as the fishing rod. The extracted PEG-peptide would then be chemically characterized by mass spectrometry. During this time, we begin to use (in an unrelated project) a new procedure (referred to as SPOTS and sold by Genosys) for synthesizing and screening defined peptides on a membrane (Frank). The disadvantage of SPOTS is that only 96 peptides could be synthesized on a membrane and screened at a time. The advantage is that the peptide in each spot has a known structure, so that the peptide showing the greatest binding did not have to be characterized, which could be a difficult task. To compensate for the lesser number of peptides that could be investigated on a SPOTS membrane in comparison with a combinatorial library, we adopted a deconvolution strategy (Han et al.; Kramer et al.). This work is in progress and should be completed in September.

SPOTS synthesis, which includes our modifications that drive the coupling reactions to completion, is done as follows.

Chemical Reagents:

1% Bromophenol Blue (100mg/10mL DMF),
Fmoc- β -Ala-OH, Fmoc-D-Arg(Pmc)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-amino benzoic acid, Arg and Lys analogs (20 different compounds),
DIPC/HOBT/DMF, Acetic Anhydride, 20% Piperidine/DMF, Methanol, DMF.

SPOTs Synthesis Procedure:

1. Prepare necessary solutions
2. Weigh out amino acids, dissolve and aliquot.
3. Thaw and label SPOTs membrane.
4. Add 0.9 mL of amino acid solution to appropriate SPOT as per schedule.
5. Incubate for 15 minutes.
6. Repeat the amino acid addition (double coupling). Incubate for another 15 minutes.
7. Wash with 3X20mL of DMF, 2 min per wash.
8. Add 800 μ L of acetic anhydride to last wash. Incubate until all blue color has gone.
9. Wash with 3X20mL of DMF, 2min per wash.
10. Add 20mL of 20% piperidine in DMF, incubate for 5 min.
11. Wash with 5X20mL of DMF, 2min per wash.
12. Add 200mL of 1% bromophenol blue to 20mL of DMF and add to membrane. Incubate until SPOTs turn blue.
13. Wash with 3X20mL of methanol, 2 min per wash.
14. Air dry using a cool air dryer.
15. Add next residue following step 4-14. If it is the last residue to be added, repeat only steps 4-7, then go directly to step 16
16. Add 20 mL of 20% piperidine/DMF to membrane. Incubate for 5 min.
17. Wash with 5X20mL of DMF, 2 min per wash.
18. Add 200mL of 1% bromophenol blue to 20mL of DMF and add to

membrane. Incubate until SPOTs turn blue.

19. Add 400mL of acetic anhydride to 20mL of DMF and add to membrane. Incubate until SPOTs turn colorless.

20. Wash with 3X20mL of DMF, 2 mins per wash.

21. Wash with 3X20mL of methanol, 2 mins per wash

22. Air dry using a cool air dryer.

23. Mix 5 mL of dichloromethane with 5mL of trifluoroacetic acid and then add 250 μ L of triisobutylsilane and add to membrane. Incubate for 1 hour.

24. Wash with 4X20mL of dichloromethane, 2 min per wash.

25. Wash with 3X20mL of DMF, 2 min per wash.

26. Wash with 4X20mL of methanol, 2 min per wash.

27. Air dry and store at -20°C. Ready for probing with HER-2/neu mRNA.

In our new strategy, we assumed that peptides containing arginine residues, more than any other amino acid, would bind avidly to RNA due to their positive charge and the ability to form the so-called "arginine fork" with phosphate groups (Calnan et al.). Lysine residues should also be used, since they have a positive charge. Also, a preference was made for using D-amino acids rather than the natural L-amino acids in order to attain peptidase resistance for the peptide. In our deconvolution strategy, the following peptides were synthesized:

D-Arg-D-Arg-D-Arg-D-Arg-D-Arg-D-Arg-D-Arg- β Ala- β Ala-membrane
and
D-Lys-D-Lys-D-Lys-D-Lys-D-Lys-D-Lys-D-Lys-D-Lys- β Ala- β Ala-membrane.

The beta-alanine residues serve as spacers to prevent steric hindrance by the membrane to incoming RNA. Surprisingly, the polylysine peptide bound HER-2/neu RNA more selectively (see below), so it was used as the basis of the deconvolution strategy. Accordingly, the first deconvolution synthesis used peptides as follows:

X-D-Lys-D-Lys-D-Lys-D-Lys-D-Lys-D-Lys- β Ala- β Ala-membrane,

where the N-terminal residue was randomized. That is, 20 different SPOTS were synthesized, and all had the same sequence except for the N-terminal residue. The 20 different amino acids used in the randomization cycle were mainly arginine and lysine analogs (RSP Inc.). Actually, duplicates of each SPOT were made (40 SPOTS total) to compare with and without N-terminal capping by acetylation. After screening with [³²P]HER-2/neu RNA in competition with tRNA (see below), the best binding peptide was identified (call it R¹) and the following set of peptides was synthesized (without capping) for the next deconvolution step.

R¹- X-D-Lys-D-Lys-D-Lys-D-Lys-D-Lys-βAla-βAla-membrane,

where the N-terminal amino acid is fixed and then the second amino acid is randomized with the 20 choices. To have greater diversity in this process, we usually selected the 2 best choices of R¹, thereby giving a total of 40 SPOTS, 20 each of:

R¹A- X-D-Lys-D-Lys-D-Lys-D-Lys-D-Lys-βAla-βAla-membrane,
and

R¹B- X-D-Lys-D-Lys-D-Lys-D-Lys-D-Lys-βAla-βAla-membrane,

where A and B are 2 different analog amino acids. In the next deconvolution cycle, the following 40 SPOTS were synthesized:

R¹-R²C- X-D-Lys-D-Lys-D-Lys-D-Lys-βAla-βAla-membrane,
and

R¹- R²D- X-D-Lys-D-Lys-D-Lys-D-Lys-βAla-βAla-membrane.

The next cycle of deconvolution would have the following 40 SPOTS:

R¹- R²-R³E- X-D-Lys-D-Lys-D-Lys-βAla-βAla-membrane,
and

R¹- R²-R³F- X-D-Lys-D-Lys-D-Lys-βAla-βAla-membrane.

After 7 deconvolution cycles, we will choose the best peptide for binding to HER-2/neu RNA as:

R¹- R²-R³-R⁴-R⁵-R⁶-R⁷-βAla-βAla-membrane,

where R¹, R², etc. may each be any one of the 20 different analogs, and its identity is known.

Then we will go through a second round of refinement by synthesizing:

X- R²-R³-R⁴-R⁵-R⁶-R⁷-βAla-βAla-membrane,

wherein the R¹ amino acid may now be different when the other 6 positions are no longer D-Lys. The other 6 positions will be similarly probed, for a total of 14 deconvolution cycles. At the time of writing of this report, we are at the step:

R¹-R²C- X-D-Lys-D-Lys-D-Lys-D-Lys-βAla-βAla-membrane,
and

R¹- R^{2D}-X-D-Lys-D-Lys-D-Lys-D-Lys-βAla-βAla-membrane.

Now that we have the processes for synthesis and screening (see below) established, we can deconvolute one position per week, and anticipate completing Tasks 1 - 4 by the end of September. We can also purchase some other amino acid analogs and make more than 20 SPOTS for each round of deconvolution.

Screening of the SPOTS membrane:

Screening of the membrane with [³²P]HER-2/neu RNA was done as follows.

Synthesis and purification of HER-2/neu mRNA:

- 1) HER-2/neu mRNA was transcribed by an In Vitro process using the Promega In Vitro transcription kit and following the protocol provided by the Protocols and Application Guide. (Promega, 1996)
- 2) Conducting a Phenol:Chloroform extraction followed by an Ethanol precipitation first purifies the transcribed HER-2/neu mRNA.
- 3) Full length HER-2/neu mRNA is separated from non-full length fragments by an 8% Urea Polyacrylamide gel. The bands are visualized by U.V. shadowing. The full-length band is excised, and separated from the gel via an electroelution.
- 4) Sample undergoes an ethanol precipitation. The sample is then lyophilized and resuspended in DEPC-treated water.

Alkaline Phosphatase and Kinase Labeling of HER-2/neu mRNA:

- 1) Dephosphorylation buffer is added to the purified HER-2/neu mRNA. The sample is preheated at 50°C for 10 minutes.
- 2) Alkaline Phosphatase is added to the HER-2/neu mRNA sample and is then placed in the 50°C H₂O for 1 hour.
- 3) The sample undergoes a phenol extraction and an ethanol precipitation. The samples are then washed with ethanol, lyophilized, and resuspended in DEPC-water.
- 4) The HER-2/neu mRNA is labeled on the 5'- end with [γ-³²P]ATP. The sample is incubated at a 37°C water bath for 30 minutes.
- 5) The HER-2/neu mRNA is purified from radioactive nucleotides by running the RNA through a Sephadex-G50 column.
- 6) The HER-2/neu mRNA is then ready to be used for hybridization with the SPOTS membrane.

Hybridization of Radioactively labeled HER-2/neu mRNA to SPOTS membrane:

- 1) SPOTS membrane is prewashed with 10 ml of PBS buffer (hybridization buffer) for 1 hour @ 30°C.
- 2) Radioactive HER-2/neu mRNA + competitor yeast-tRNA are added to the hybridization buffer.

- 3) Hybridization time is for 2 hours at 30°C.
- 4) Hybridization buffer is poured off and residual counts are quantified via Scintillation counting.
- 5) The SPOTs membrane is then washed with 10 ml of hybridization buffer for 1 hour @ 30°C. The hybridization buffer is poured off and the SPOTs membrane is exposed to a Phosphorimaging screen. for a specified period. The imaging screen is then removed and quantified by using molecular imaging software.
- 6) Radioactively bound HER-2/neu mRNA to the SPOTs membrane is removed by washing the SPOTs membrane with 10 ml of 6M Guanidinium HCl for 1 hour @ 30°C. The SPOTs membrane is then ready to undergo another hybridization.

Results of SPOTS screening:

The binding of [^{32}P]HER-2/neu RNA to each SPOTS membrane is depicted in Figures 1 - 8. In order to observe differential binding, a critical factor to determine is the ratio of competitor (unlabelled) yeast tRNA to [^{32}P]HER-2/neu RNA; the amount of peptide per SPOT is not known exactly, but is constant from one SPOT to the other on both the same membrane and on different membranes. The protocol should be designed to reveal the peptide which binds to HER-2/neu RNA when other RNAs are in tremendous excess, as would be the situation in a cancer cell. In Figures 1-4, the amount of competitor tRNA is increased in the steps: 28 ng, 0.1 mg, 1.3 mg and 13.2 mg. Whereas discrimination among the different peptides for amount of [^{32}P]HER-2/neu RNA bound is seen at every level of competitor tRNA, 3 SPOTS in particular predominate at a competitor excess of 10^8 (Figure 3) and even more so at an excess of 10^9 .

This experiment, which preceded and helped develop the deconvolution paradigm, may be explained as follows. As mentioned above, each peptide was based on variations of a *DArg7* platform. Three negative control peptides comprising the sequence *DLys7* were also made on the same membrane. Surprisingly, the strongest binders of HER-2/neu RNA under the high stringency conditions (Figures 3 and 4) were the lysine peptides. The differences in these lysine peptides is the linker attaching the peptide to the membrane, the best choice of which was observed to be two residues of beta-alanine. Another purpose of this experiment was to evaluate the effect of placing a sterically constrained amino acid at different positions in the peptide backbone. Accordingly, the 3 isomers of aminobenzoic acid (ortho, meta and para; B2, B3 and B4) were placed at various positions in the peptide sequence, as indicated in the figures. Whereas these different rigid bends in a *DArg7* background did not appear to substantially effect binding of RNA, the aminobenzoic acid isomers will be tested once again in the deconvolution process. However, this experiment caused us to switch to the *Lys7* background sequence for the deconvolution process.

Deconvolution of the N-terminal amino acid is shown in Figures 5 and 6, at two different levels of competitor tRNA. As seen in Figure 6, the two best peptides (see checkmarks on Figure 6) have as the N-terminal amino acid, L-Ala-4-Pip(N-amidino) and L-hLys with the N-terminus nonacetylated. [Note that the peptides on the left are nonacetylated and those on the right are acetylated with the duplicate sequences.] For deconvolution of the next amino acid, position 1 (N-terminal residue) was either Ala-4-Pip(N-amidino) (left side of Figure 7) or hLys (right side of Figure 7). The best peptides (open bars in Figure 7) have at their second position either L-trans-cha(4-CH₂NH₂) or (s)-2-amino-4-[(2-amino)pyridimyl-butanoic acid]; both have hLys at position 1. In the third cycle of deconvolution, the peptides will have the following characteristics. The N-terminal is not acetylated (free amino group), the N-terminal residue (position 1) is L-hLys and the second amino acid is either L-trans-cha(4-CH₂NH₂) (one set of 20 peptides) or (s)-2-amino-4-[(2-amino)pyridimyl-butanoic acid] (another set of 20 duplicate peptides). Position 3 is now randomized.

Task 5:

We separately developed a procedure for synthesizing oligonucleotide-peptide conjugates. The problem that had previously hampered the solution phase coupling reaction was that the positively charged peptide and the negatively charged oligonucleotide would precipitate rather than react chemically. In the new procedure, the oligonucleotide is synthesized with a 5'-amino group. The amino group is left unprotected, but the oligonucleotide is left fully base-protected on the solid support. The amino group is then activated by reaction with bromoacetic acid, which is then reacted with a cysteine containing peptide.

When deconvolution is completed, this peptide will be:

R¹-R²-R³-R⁴-R⁵-R⁶-R⁷-βAla-βAla-Cys,

where the C-terminal cysteine residue occupies the position of the membrane and, therefore, does not participate in the binding of HER-2/neu RNA. In our development experimentation, we used an arginine-containing peptide as a model. In either case, the peptide to be coupled is synthesized on a solid support, cleaved from the support and deprotected, purified by HPLC and then conjugated. The next step is cleavage of the peptide-oligonucleotide conjugate from the solid support with simultaneous deprotection of the bases. By using fast-release monomers for oligonucleotide synthesis and milder ammonia cleavage conditions, we were able to obtain the peptide-oligonucleotide product. Further details on this process will be given in next year's report after it is applied to the fully deconvoluted peptide.

This product would then be used in the RNase H cleavage assay to determine the site of hybridization on the target. We have successfully initiated RNase H assays of HER-2/neu RNA, as reported last year.

KEY RESEARCH ACCOMPLISHMENTS

SPOTS peptide synthesis has been established and improved.

Screening of SPOTS membranes with HER-2/neu RNA has been optimized.

Our novel deconvolution process for identifying a peptidic compound having specificity of binding to HER-2/neu mRNA appears to be working.

A process for synthesizing conjugates of oligonucleotides and cationic peptides has been developed.

REPORTABLE OUTCOMES

None,

CONCLUSIONS

The first and perhaps major technological challenge is to design a peptidic compound that is capable of binding to a specific folded domain on a target RNA, in this case HER-2/neu mRNA, with high selectivity. There are many possible approaches, each of which includes synthesis and screening. After evaluating several different approaches, we believe that we have chosen one that is well suited to our expertise and to the level of financial support of this project. The peptidic compound we expect to have selected this fall should at least be sufficient for proof-of-principle of our new strategy required to develop a therapeutic agent for breast cancer in which HER-2/neu is overexpressed. Even if it is not suitable as the therapeutic agent, it should be a good model from which to further develop the desired therapeutic product. The first 3 rounds of deconvolution indicate a significant but not yet substantial degree of binding specificity, even though fewer than half of the oligolysine platform sequence has been deconvoluted so far. Once we complete this deconvolution process, we will be able to evaluate whether we have successfully overcome this first challenge. The next phase of the project dealing with RNase H cleavage of HER-2/neu mRNA should be less of a technological challenge, since we have developed and tested the necessary methodology.

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Kramer, A., Schuster, A., Reineke, U., Malin, R., Volkmer-Engert, R., Landgraf, C. and Schenider-Mergener, J. (1994) *METHODS* 6, 388-395.

Figure 1:

Peptide Binding Assay: Peptide vs. HER2/neu mRNA 25.6pg of HER2/neu mRNA + 28ng of yeast t-RNA

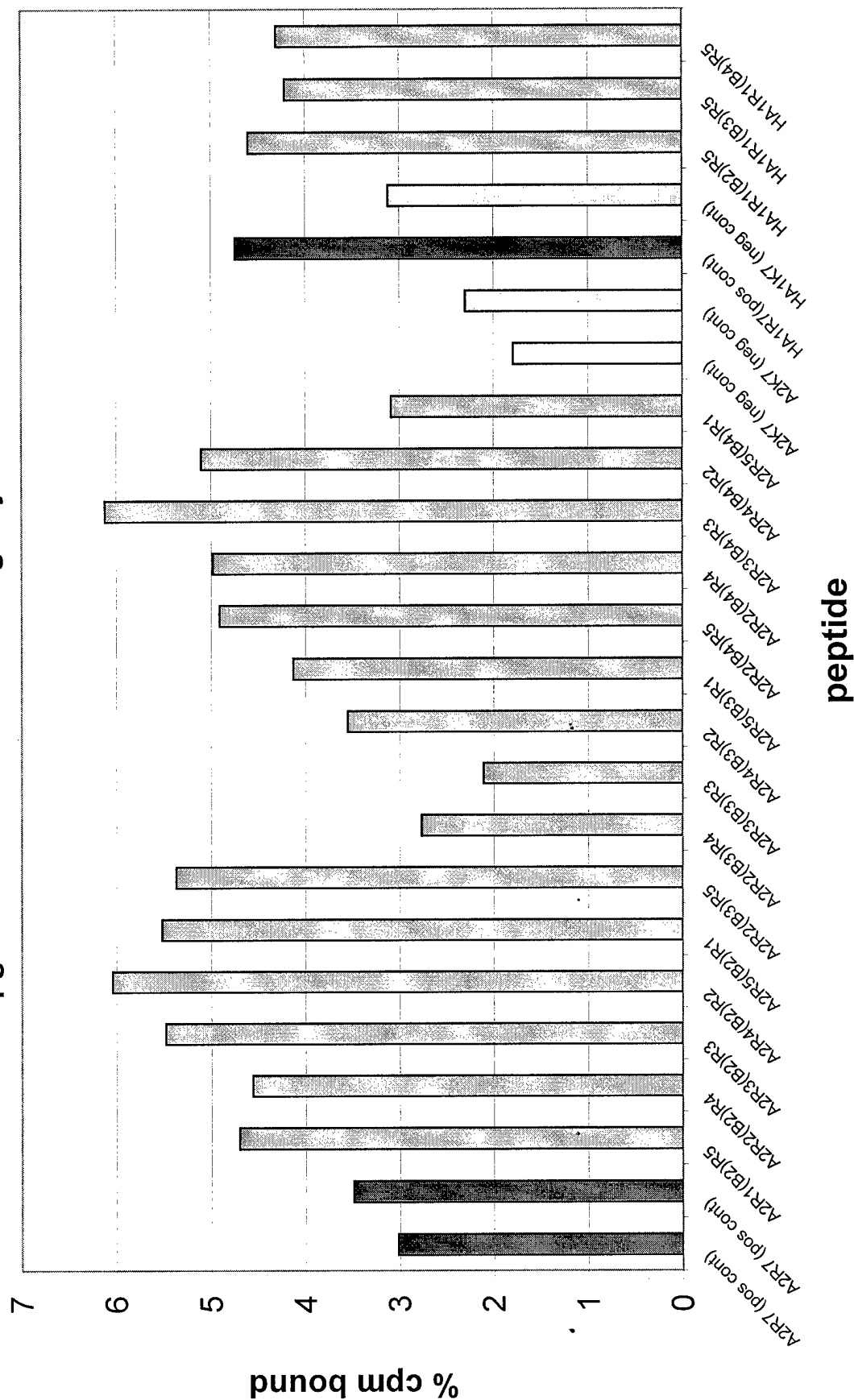
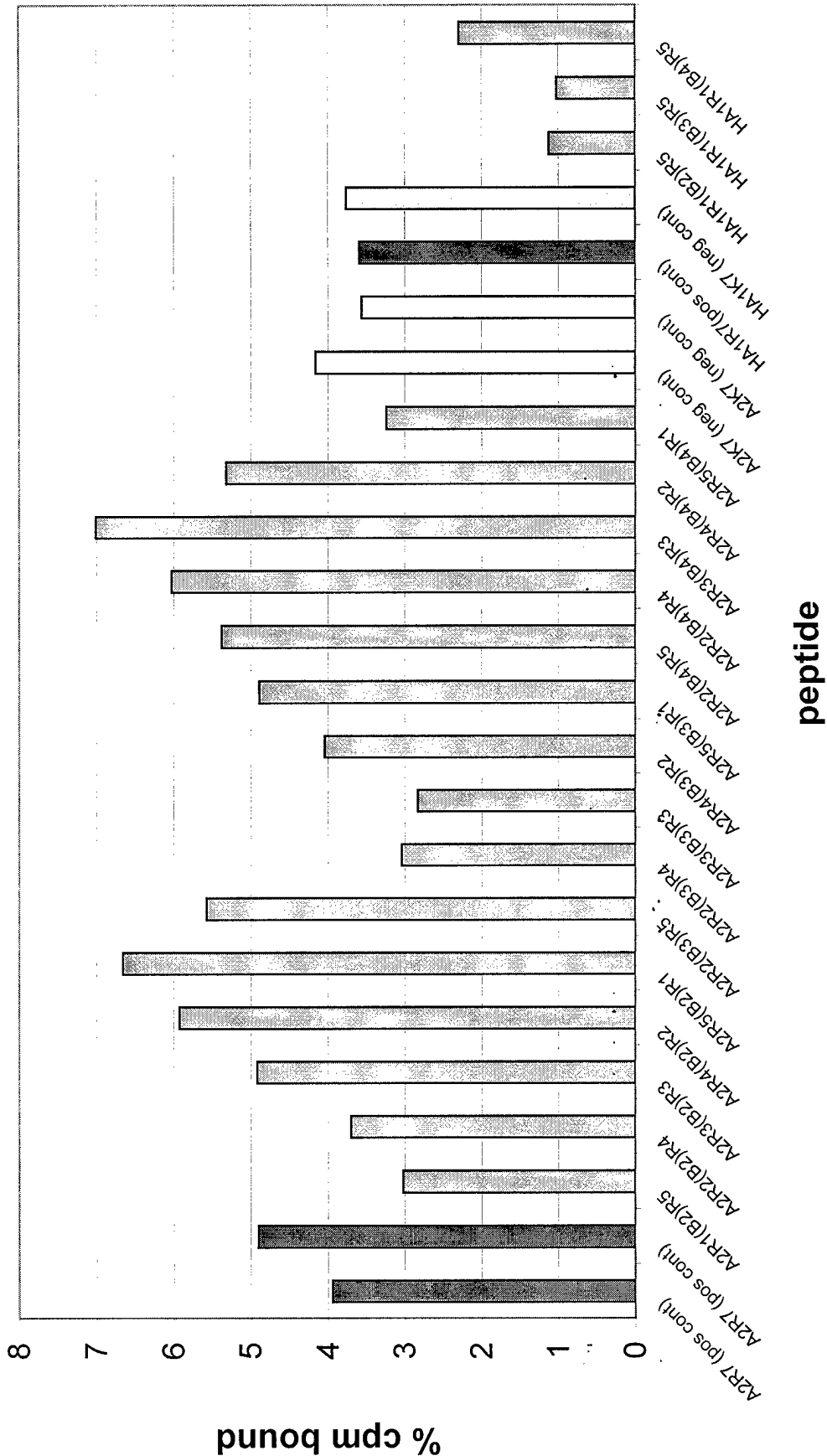


Figure 2:

Peptide Binding Assay: pepetide vs. HER2/neu mRNA
25.6pg of HER2/neu mRNA + 100ug yeast t-RNA



Peptide Binding Assay: peptide vs. HER2/neu mRNA
13.2pg HER2/neu mRNA + 1.3mg yeast t-RNA

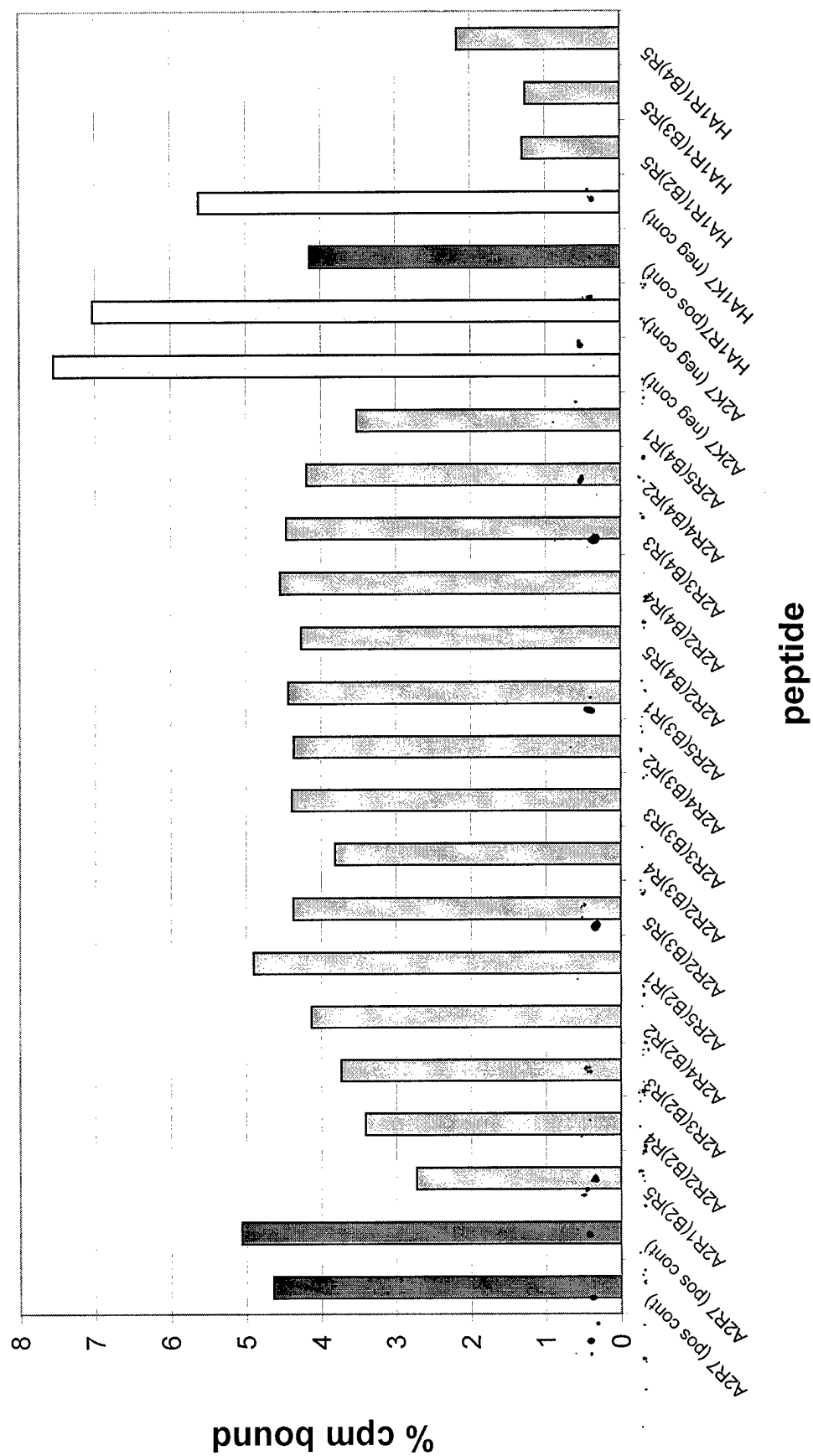
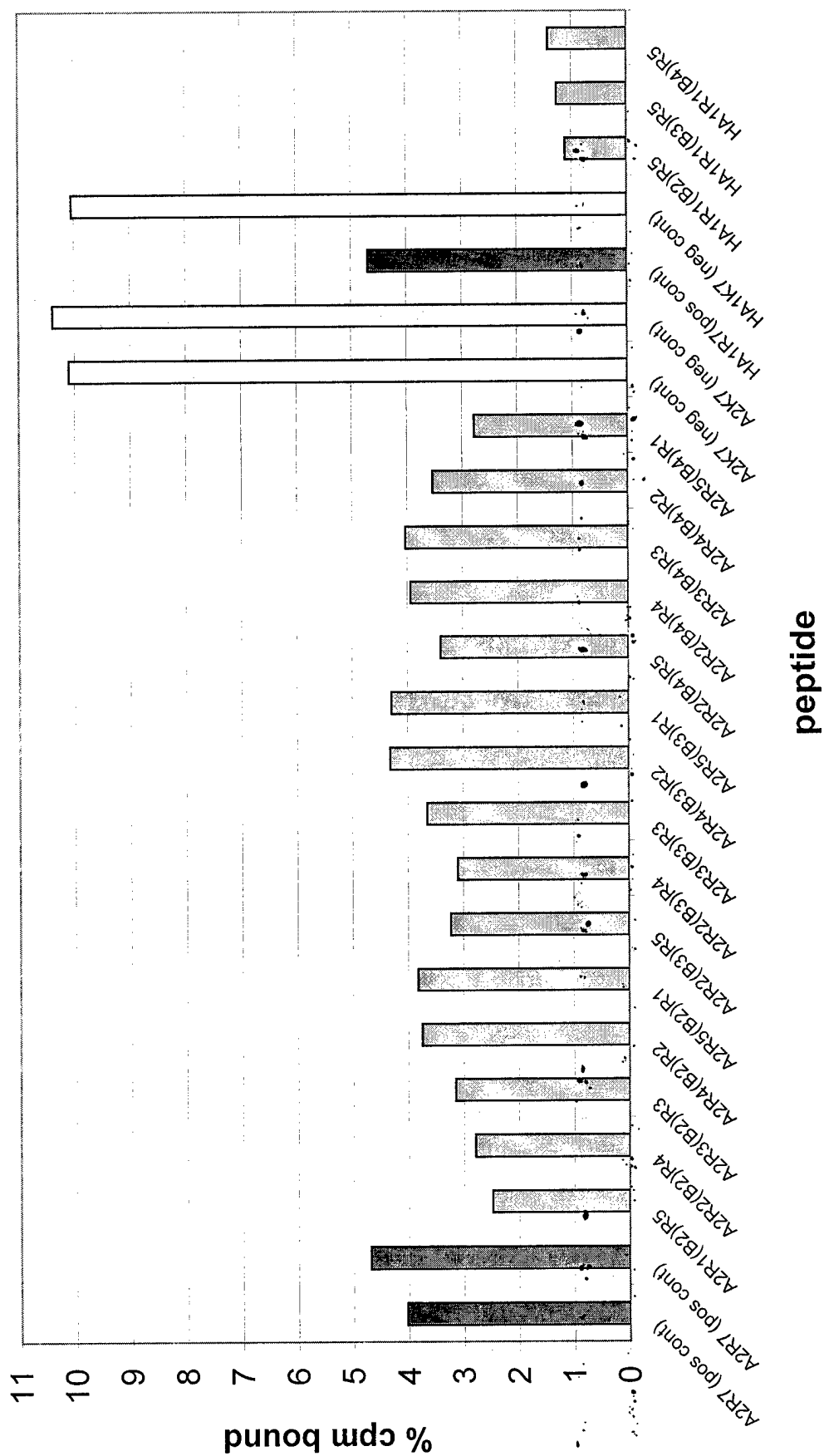


Figure 4:

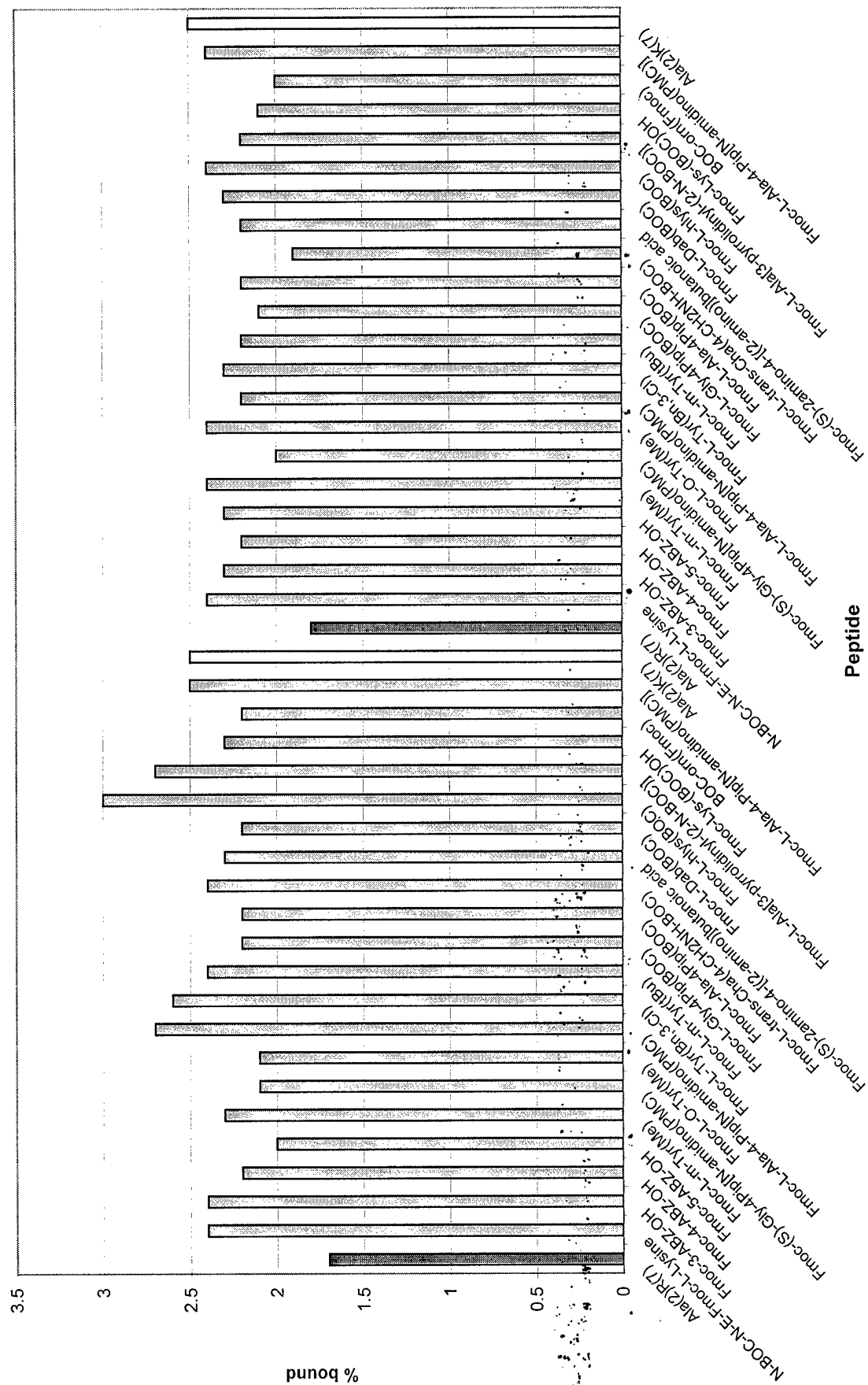
Peptide Binding Assay: Peptide vs. HER2/neu mRNA

13.2 pg HER2/neu mRNA + 13.2mg yeast t-RNA



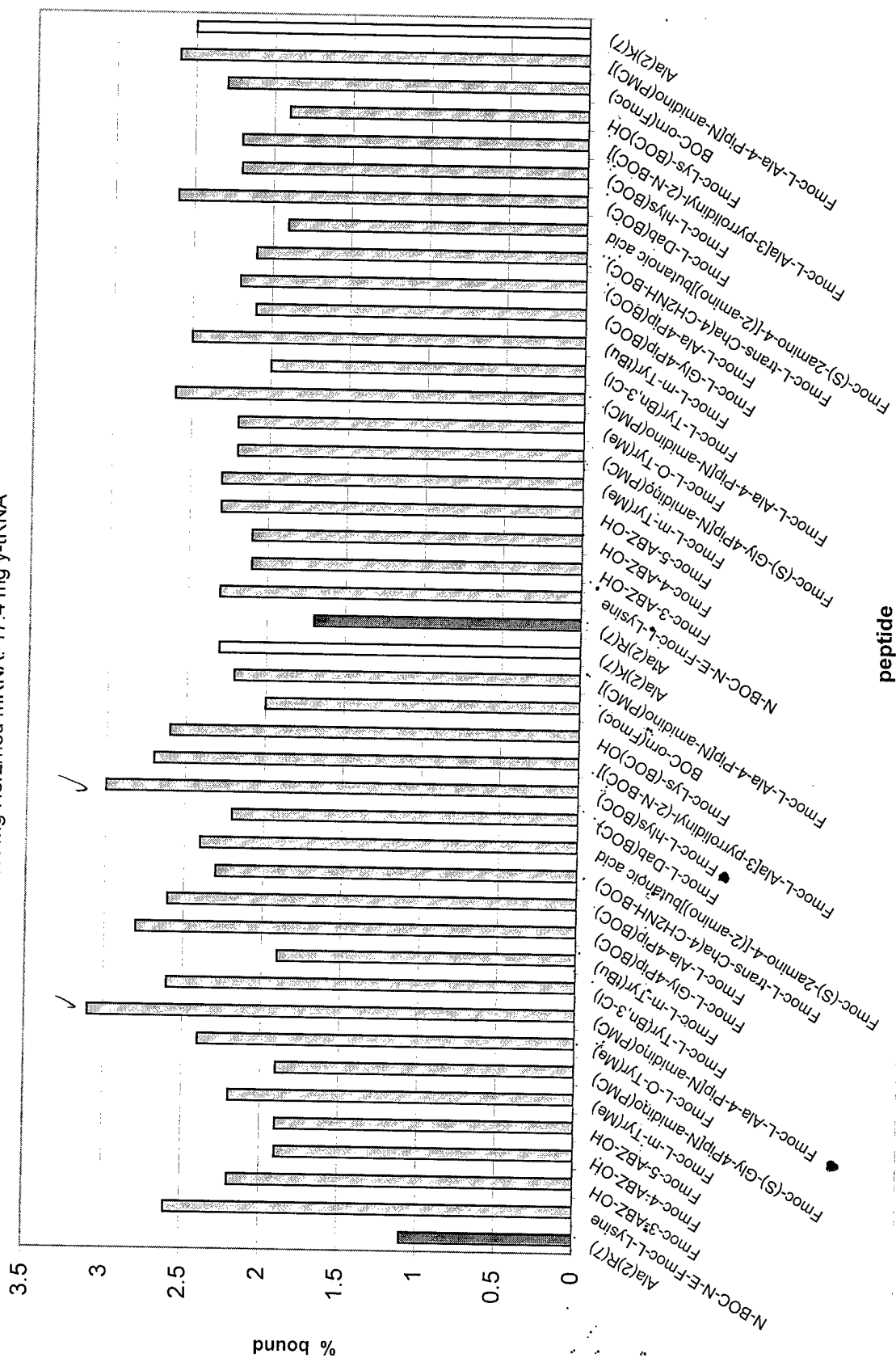
Peptide Binding Assay: Peptide vs. Her2/neu mRNA

87.1ng Her2/neu mRNA: 8.71mg y-tRNA



5

17.4ng Her2/neu mRNA: 17.4 mg y-tRNA



Peptide Binding Assay: peptide vs. HER2/neu mRNA
871ng HER2/neu mRNA + 25mg y-tRNA

